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Transformation of BALB/c-3T3 Cells: I. Investigation of Experimental Parameters that Influence Detection of Spontaneous Transformation

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The frequency of spontaneous morphological transformation is an important variable in measuring chemical-induced transformation in BALB/c-3T3 clone A-31-1-13 cell cultures. Data from 110 experiments, which included benzo[*a*]pyrene control groups and other chemical treatment groups, were analyzed for factors that influenced spontaneous transformation. Spontaneous transformants demonstrated a continuum of morphological variants (type I, II, and III foci) that fit a normal distribution if converted to $\log_{10}$. The magnitude of transformation depended on the ampule of cryopreserved cells and the serum lot. Although the average frequency was approximately 0.71×10^{-6} (type III foci/cell that survived and proliferated to confluence), the absolute number of foci/vessel increased in proportion to the surface area of the culture vessel. Thus, the frequency of spontaneous transformation was directly related to the cumulative number of mitoses that occurred in forming the contact-inhibited monolayer. These data are consistent with a hypothesis that spontaneous transformation in BALB/c-3T3 cells is a mutational event or some other single-step phenomenon.

Introduction

Spontaneous morphological transformation of cultured mammalian cells is a relatively rare event in some cell transformation assay systems; however, it is easily detected in other systems. For example, the frequency of spontaneous type II or type III foci in the C3H10T1/2 cell transformation assay has been either low or undetectable (*1–5*). Similarly, many laboratories have not reported spontaneous transformed colonies of Syrian hamster embryo (SHE) cells in the SHE colony transformation assay (*6,7*); however, other laboratories report background activities for this assay (*8*). In contrast, spontaneous transformation has been routinely detected in both the BALB/c-3T3 (*9–13*) and Simian adenovirus 7/SHE (SHE/SA7) transformation assays (*14-16*). The mechanism of spontaneous transformation in the SHE/SA7 system has been correlated with the insertion of viral DNA into the genome of the host cells (*15*). In contrast, the mechanism of spontaneous transformation of BALB/c-3T3 cells is not understood (*17*).

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The standard assay design for the BALB/c-3T3 cell transformation assay (*11,18–21*) involves seeding 25-cm^2 flasks or 60-mm dishes with 1×10^4 cells. At this seeding density, Dunkel et al. have reported (*9*) an average frequency of spontaneous transformation of 0.25 type III foci/vessel for the 1-13 clone of A31 BALB/c-3T3 cells. A comparable spontaneous frequency was observed in this laboratory for the same clone of cells for 260 experiments conducted over a 5-year period (*11*). Nevertheless, the range of spontaneous transformation frequencies in individual experiments was very large in both of these laboratories, and this variability was attributed to both the source of fetal bovine serum (FBS) used in culture medium (*11-12*) and to the passage number of laboratory cultures (*22*).

This investigation was conducted to determine additional experimental parameters that caused variable detection of spontaneous transformation of BALB/c-3T3 cells. To eliminate parameters that are known to affect the frequency of spontaneous transformation, a single pool of cryopreserved cells was used in all experiments. Likewise, all experiments used laboratory cultures of BALB/c-3T3 cells that were maintained in log-phase growth by biweekly passage at low seeding densities. This procedure was performed to prevent spontaneous transformed cells from having a selective growth advantage over wild type (WT) cells in a confluent, contact-inhibited cell culture. The two lots of FBS used in this study were screened for

their capacity to support comparable levels of spontaneous transformation. Finally, because cultures of BALB/c-3T3 cells always contained a continuum of different sizes of type I, II, and III (I–III) foci, variability among spontaneous transformation frequencies might be related to the inherent subjectivity of scoring individual morphological variants; thus, all foci observed with an acceptable size were recorded.

Materials and Methods

Cell Culture

These investigations used the 1-13 clone of A-31 BALB/c-3T3 cells, and the cells were a gift from T. Kakunaga (*23,24*). The materials and methods used to culture BALB/c-3T3 cells have been reported in detail (*11*). Briefly, cells were cultured in Eagle's minimal essential medium [EMEM; Gibco, Grand Island, NY, or Hazleton Research Products (HRP), Denver, PA] supplemented with 100 μg/mL streptomycin, 50 μg/mL gentamicin, 292 μg/mL L-glutamine (Quality Biologicals, Gaithersburg, MD), and a selected lot of heat-inactivated (50°C/1 hr) FBS (Armour Biochemicals, Kankakee, IL or HRP). The three criteria used to select an FBS lot included its ability to support a high cloning efficiency of the cells (≥ 35%), a low but consistently detectable frequency of spontaneous transformants (~0.50 type III foci/vessel), and a high benzo[*a*]pyrene (BaP)-induced transformation response [about five type III foci/vessel for a 48-hr exposure to 0.2 μg/mL BaP). Cell cultures were seeded in culture medium containing 10% v/v FBS and were maintained with maintenance culture medium containing 7.5% v/v FBS. All cell cultures were incubated in a water-saturated, 4.5–5.0% CO_2/air atmosphere at 36 to 37°C. Laboratory cultures of cells were maintained in log-phase growth and were not permitted to become confluent. They were passaged biweekly using 5×10^4 cells/100-mm culture dish (Corning Science Products, Corning, NY) and were used between passages 4 (p4) and p24. Early passage laboratory cultures were tested and found to be mycoplasma-free.

Transformation Assay

Spontaneous transformation of BALB/c-3T3 cells was evaluated in a standard transformation assay protocol that has been reported in detail (*11*) and is summarized in these investigations in part IV of this series (*25*). In each experiment spontaneous transformation was detected in the negative control that consisted of 40–80 vessels seeded with 3.2×10^4 cells/vessel. The negative control included either untreated cultures or solvent control cultures treated with a noncytotoxic concentration of the solvent vehicle used for the test chemicals. Solvent control treatments were applied to cell cultures for 48-hr, days 2–4, using standard procedures (*25*). Spontaneous transformation was also detected in research experiments using different numbers of cells seeded into different-size culture vessels that included 35-mm, 60-mm, 100-mm (Corning), or 150-mm (Falcon Plastics, Cockeysville, MD) dishes, as well as 25-cm^2 culture flasks (Corning).

Evaluation of Transformed Foci

The method used to evaluate transformed foci of BALB/c-3T3 cells has been reported in detail (*11*) and summarized in part IV of this series (*25*). Briefly, the number of type I–III transformed foci of BALB/c-3T3 cells were identified microscopically using published criteria (*5,11,19–21,26*), and type III foci had three phenotypic properties, including piling and overlapping cells, disorientation of cells at the periphery of the focus, and invasion of transformed cells into a contact-inhibited monolayer of WT cells. Type I and II foci also appeared in many different sizes, but they lacked one or more of the three phenotypic properties of the type III transformed focus.

Statistical Methods

The appearance of transformed foci of BALB/c-3T3 cells in sets of culture vessels was abnormal in its distribution, and large numbers of foci were routinely observed in a few culture vessels of a set (*11,27–28*). After examining several mathematical transformations (*29*), the focus data were found to fit a logarithmic ($\log_{10}$) transformation (*11,27*). This method involved adding one to the number of transformed foci scored in each vessel, converting this total to a $\log_{10}$ equivalent value, and then determining the mean transformation $\log_{10}$ response in each treatment set. The presence of outlier observations among the data sets were determined using standard statistical procedures (*30*).

The significant difference between spontaneous transformation frequencies was determined using SAS software (*31*). An analysis of variance was performed on $\log_{10}$ data using the F-test, and the significant difference between responses was calculated using modifications of the Student's t-test that assumed either equal variance (EV) or unequal variance (UV) between the control and the comparison set. The correct t-statistic was distinguished by a F-test confidence level of 5% (i.e., $p<0.05$). Finally, the probability level of individual sets of data having a significant activity was determined using the appropriate UV or EV t-statistic.

Results

Mathematical Distribution of Spontaneous Transformants of BALB/c-3T3 Cells

The number of types I, II, and III foci of BALB/c-3T3 cells that arose spontaneously in culture vessels was variable. In most experiments the majority of vessels had either few foci or no foci; however, there were usually a few vessels that had large numbers of foci. To determine the mathematical distribution of the transformation phenotype, all foci observed in 37 experiments conducted over a 6-month period were tabulated according to the number of foci per vessel. Approximately 55% of 2865 culture

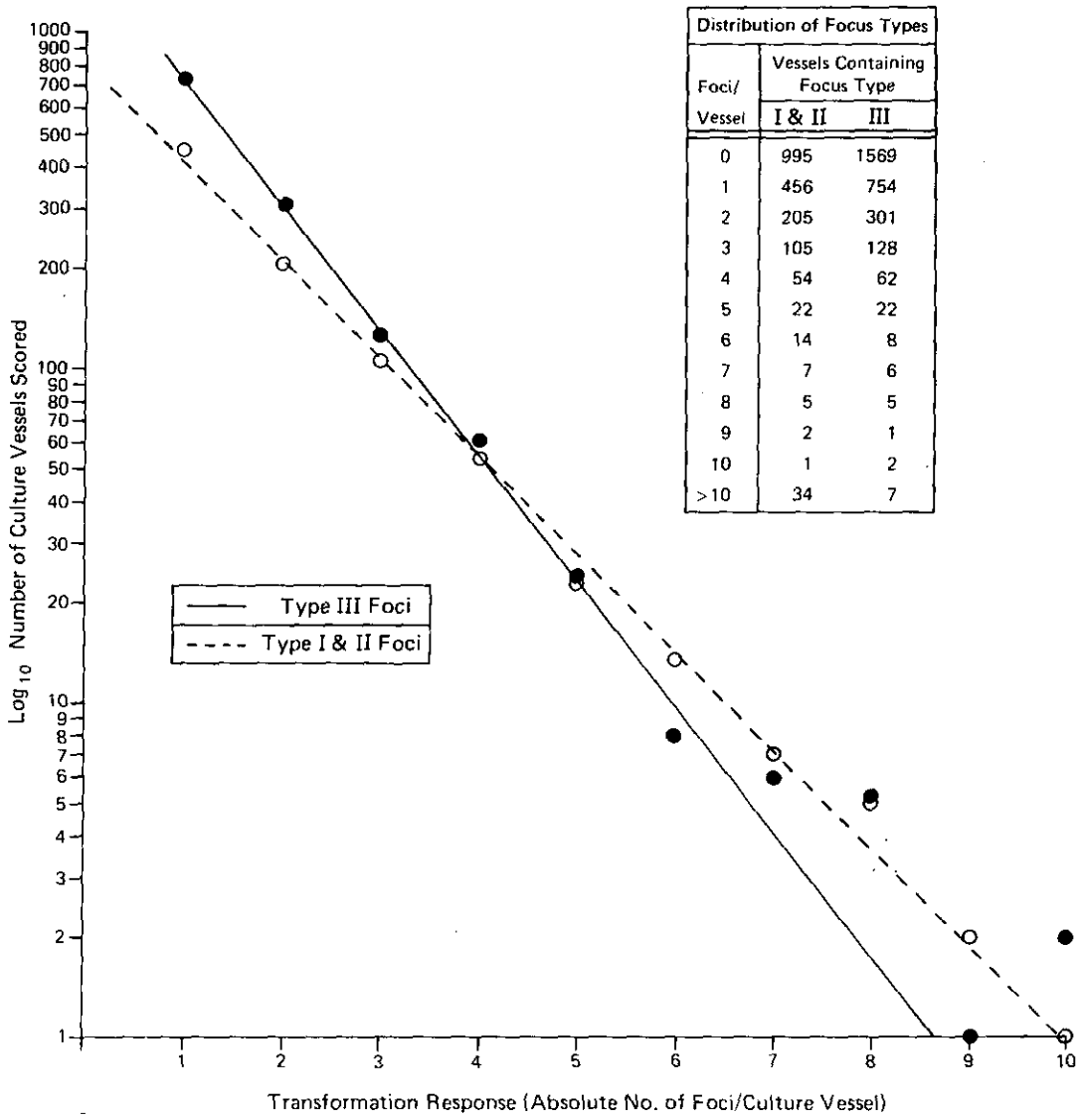

FIGURE 1. Mathematical distribution of transformed foci of BALB/c-3T3 cells. A total of 2873 culture vessels in 37 consecutive experiments were evaluated for the presence of type III foci ≥ 2 mm in diameter, as well as type I and II foci ≥ 1 mm in diameter. The total number of culture vessels with different numbers of foci were tabulated, and the data were examined for their fit to different mathematical distributions. The data were demonstrated to fit a $\log_{10}$ distribution, and they are plotted on a $\log_{10}$ scale.

vessels in these experiments had no type III foci ≥ 2 mm in diameter, and 37% of the vessels had only 1 or 2 foci. Of the remaining 241 culture vessels, 234 vessels had 3–10 type III foci/vessel, and 7 vessels had 11 or more foci. This skewed distribution of type III foci was shown to fit a $\log_{10}$ mathematical distribution, and conversely, the $\log_{10}$-transformed data had a normal distribution (Fig. 1).

Analyses of the combined type I and II focus data were also performed in the same 37 experiments. The appearance of type I–II foci fit a $\log_{10}$ distribution, and roughly half (52.4%) of the 2865 culture vessels had no type I and II foci ≥ 1 mm in diameter, and 34.2% of the vessels had either 1 or 2 foci/vessel. The remaining 12.9% culture vessels had either 3–10 foci/vessel (11.1%) or >11 foci/vessel (1.8%).

Affect of Different Experimental Parameters on Spontaneous Transformation

The affect of five different experimental parameters on the magnitude of spontaneous transformation frequencies of BALB/c-3T3 cells was investigated in a total of 110 experiments. The experimental parameters that were variable included *a*) 18 ampules of cryopreserved cells from a single pool; *b*) three seeding densities of 0.32, 1.0, and 3.2×10^4 cells/vessel; *c*) two FBS lots; *d*) p4–p22 passage levels of cultures; and *e*) four culture vessel surface areas. Analyses of the first three of these experimental parameters were compared by pooling experiments that used the same ampule of cells (refer to Table 1). In addition, experiments numbered 1–61 that used FBS lot A and ampules of

Table 1. Affect of seeding density on frequencies of spontaneous transformation of cells derived from 18 ampules of cells.[a]

Experiment parameter[b]		Frequency of spontaneous transformation,[c] mean ± SE foci/vessel						
		Type I–III focus frequency[d]			Type III frequency[d]			
Amp. no.	FBS lot	0.32[e]	1.0	3.2	0.32[e]	1.0	3.2	*n*
1N[f]	B	13.0 ± 2.85	9.11 ± 2.53	9.16 ± 2.19	5.63 ± 1.30	4.22 ± 1.34	4.63 ± 1.11	6
1P	B	3.42 ± 0.91	1.85 ± 0.54	3.05 ± 1.35	1.13 ± 0.33	1.16 ± 0.40	1.25 ± 0.55	4
1G	A	3.22 ± 0.70	2.02 ± 0.45	3.02 ± 0.70	1.22 ± 0.26	0.87 ± 0.15	1.27 ± 0.27	8
1F	A	3.02 ± 0.82	3.02 ± 0.70	5.72 ± 1.36	0.62 ± 0.11	0.36 ± 0.06	0.56 ± 0.15	5
1J	A	2.89 ± 1.27	2.44 ± 0.85	4.73 ± 2.52	0.79 ± 0.29	0.88 ± 0.22	1.49 ± 0.65	3
1R	B	2.30 ± 0.80	2.18 ± 0.91	5.26 ± 1.64	0.58 ± 0.20	0.65 ± 0.18	1.72 ± 0.44	5
1M	B	2.26 ± 0.30	1.02 ± 0.29	1.52 ± 0.34	0.91 ± 0.15	0.42 ± 0.09	0.78 ± 0.20	7
1Q	B	1.43 ± 0.31	0.91 ± 0.15	1.03 ± 0.13	0.74 ± 0.13	0.50 ± 0.13	0.58 ± 0.08	9
1O	B	1.26 ± 0.32	0.74 ± 0.22	0.87 ± 0.23	0.71 ± 0.22	0.49 ± 0.17	0.57 ± 0.15	10
1I	A	1.23 ± 0.15	0.49 ± 0.11	1.03 ± 0.11	0.56 ± 0.25	0.25 ± 0.08	0.46 ± 0.04	5
1E	A	1.18 ± 0.19	0.96 ± 0.17	1.24 ± 0.20	0.44 ± 0.09	0.52 ± 0.13	0.51 ± 0.13	8
1H	A	1.18 ± 0.21	0.80 ± 0.24	1.41 ± 0.32	0.58 ± 0.10	0.25 ± 0.06	0.46 ± 0.03	9
1A	A	ND	ND	ND	0.52 ± 0.15	0.43 ± 0.13	0.98 ± 0.30	4
1B	A	ND	ND	ND	0.35 ± 0.16	0.25 ± 0.09	0.26 ± 0.11	4
1K	A	0.82 ± 0.16	0.52 ± 0.15	0.72 ± 0.26	0.33 ± 0.06	0.23 ± 0.05	0.31 ± 0.08	7
1D	A	0.78 ± 0.30	0.46 ± 0.05	0.44 ± 0.10	0.29 ± 0.08	0.18 ± 0.03	0.19 ± 0.02	4
1L[f]	B	0.63 ± 0.13	0.51 ± 0.21	0.83 ± 0.05	0.38 ± 0.08	0.23 ± 0.05	0.43 ± 0.22	7
1C[f]	A	0.22 ± 0.07	0.21 ± 0.05	0.25 ± 0.08	0.09 ± 0.03	0.16 ± 0.06	0.17 ± 0.07	3
Median frequencies		1.26	0.91	1.24	0.58	0.43	0.57	

Abbreviations: Amp. no., ampule number; FBS lot, fetal bovine serum lot; n, number of experiments; ND, *not determined.*

[a]To facilitate comparison of experimental parameters of ampule aliquot number and cell seeding density with frequencies of spontaneous transformation, the average transformation frequencies for cell cultures derived from individual ampules of cells were rank-ordered. The ampule with *the highest average frequency of type I–III foci was presented first at the* 0.32 × 10^4 *cells/vessel seeding density, and the ampule with the lowest* frequency was presented last. Information on individual experiments is provided in Table A1.

[b]All of the cells in this investigation were derived from different ampules of cells in one large pool of cryopreserved cells. The first ampule used was designated 1A. The number of experiments (*n*) set up with laboratory cultures from each ampule is in parentheses. The source of FBS lot A and B are provided in Materials and Methods.

[c]Due to the $\log_{10}$ distribution of the spontaneous transformed foci detected in these experiments (Fig. 1), the transforming activity of each culture vessel was first mathematically transformed to the $\log_{10}$ before the transformation frequency of individual seeding density sets of cultures was calculated. Thus, the mean number of transformed foci in this table is the anti-log of the $\log_{10}$ mean transformation frequency.

[d]Spontaneous transformation of BALB/c-3T3 cells resulted in a continuum of type I, II, and III foci of different sizes. The type I–III transformation frequency in this investigation included type I and II foci ≥ 1 mm in diameter and type III foci ≥ 2 mm diameter. The contribution of type I and II foci alone can be calculated by subtracting the frequency of type III foci from the frequency of type I–III foci.

[e]The seeding density is given as × 10^4 cells/vessel. Laboratory cultures of different passage levels were trypsinized and cells were replated at seeding densities of 0.32, 1.0, and 3.2 × 10^4 cells/60-mm dish (or 25-cm^2 flask).

[f]Outlier experiment. An outlier experiment in this investigation was defined as an experiment with a frequency of spontaneous transformation that was significantly different from other experiments that used cells from laboratory cultures from the same ampule (*27*). Three of the 110 experiments were determined to be outliers, including *a*) experiment 8 using cells from ampule 1C, *b*) experiment 62 using cells from ampule 1L, and *c*) experiment 77 using cells from ampule 1N (refer to Table A1).

cells 1A–1L were separated from experiments 62–110 that used FBS lot B and ampules of cells 1L–1R. The effect of the remaining two experimental parameters of cell passage level and cell surface area on spontaneous transformation frequencies are discussed in Table A1, and Figure 2, respectively.

Ampule of Cryopreserved Cells. The detection of high and low spontaneous transformation frequencies of BALB/c-3T3 cells was shown to be highly correlated with experiments that used cells derived from different cryopreserved ampules. For example, at the lowest seeding density of 0.32 × 10^4 cells, cells from ampule 1N had an average frequency of type I–III foci of 13.0 foci/vessel, and this frequency was about 59-fold higher than the average frequency of 0.22 foci/vessel of cells from ampule 1C (Table 1). Similarly, at the same seeding density, cells from ampule 1N had an average frequency of type III foci of 5.63 foci/vessel, which was about 63-fold higher than the average frequency of 0.09 foci/vessel of cells from ampule 1C (Table 1). Comparable differences in type I–III and III transformation frequencies were also noted for ampules 1N and 1C in experiments that were seeded at 1.0 and 3.2 × 10^4 cells/vessel.

Initial Seeding Density. Variability of average spontaneous transformation frequencies of BALB/c-3T3 cells was observed to be essentially independent of the initial seeding density of the cells. The average frequency of type I–III foci of cells from ampule 1N seeded at densities of 0.32, 1.0, and 3.2 × 10^4 were 13.0, 9.09, and 9.16 foci/vessel, and the average frequency of type III foci for the same seeding densities were 5.63, 4.22, and 4.63 foci/vessel (Table 1). Similarly, the frequencies of type I–III foci of cells from ampule 1C seeded at densities of 0.32, 1.0, and 3.2 × 10^4 cells/vessel were 0.22, 0.21, and 0.25 foci/vessel, and the frequencies of type III foci were 0.09, 0.16, and 0.17 foci/vessel (Table 1). Thus, ampules of cells with an approximately 60-fold difference in the average spontaneous transformation frequency had less than a 1.5-fold dif-

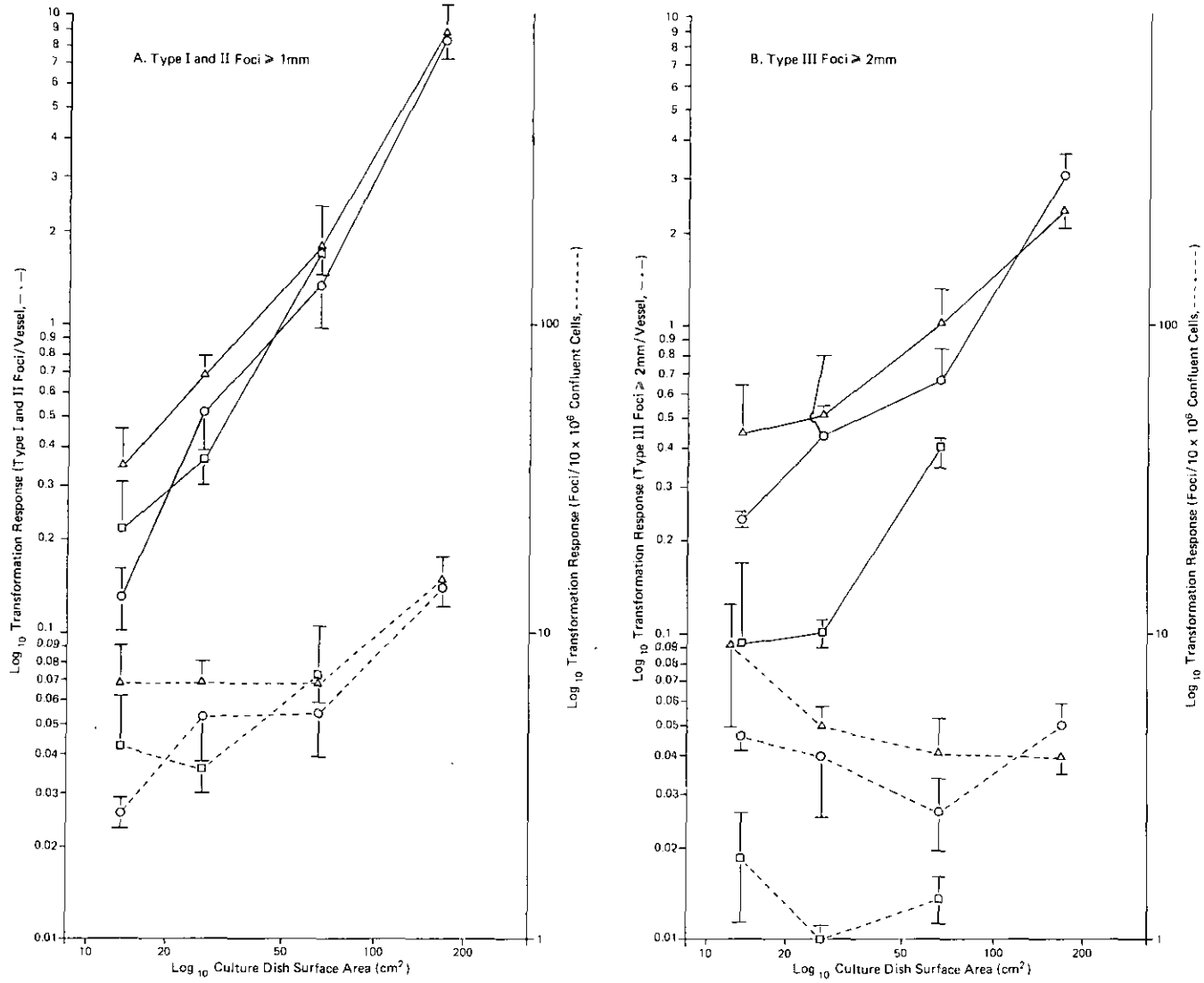

FIGURE 2. Effect of culture vessel surface area on frequency of spontaneous transformation of BALB/c-3T3 cells. The effect of culture vessel surface area on the frequency of spontaneous transformation of BALB/c-3T3 cells was examined in three different independent experiments: no. 1 (□), no. 2 (○), and no. 3 (△). See Materials and Methods for experimental details. (*A*) Type I–II foci, (*B*) transformed type III foci. The transformation frequencies were calculated using $\log_{10}$ transformed foci/culture vessel (solid lines) and $\log_{10}$ transformed foci/10 $\times$ 10^6 confluent cells (dashed lines).

ference in the average transformation frequency for cells seeded at different densities.

FBS Lot. The lot of FBS used in the transformation experiments had a minor effect on the magnitude of the frequency of spontaneous transformation. The median type I–III frequency of experiments using serum lot B was 2.26 foci/vessel versus 1.18 foci/vessel for lot A. Thus, the median frequency of spontaneous transformation detected in experiments using FBS lot B was about 2-fold higher than the frequency detected using FBS lot A. The relatively small effect of FBS lot on the frequency of spontaneous transformation was expected because the two FBS lots had been screened and had comparable activities in preliminary experiments.

Culture Passage Level. Spontaneous transformation frequencies detected in experiments using cells with different passage levels are compared in Table A1. The data were rank-ordered from the ampule of cells, resulting in the highest average frequency of type III foci to the ampule with the lowest average frequency. In addition, the data from individual experiments/ampule were rank-ordered. The experiment with the highest type III response at the highest seeding density of 3.2 $\times$ 10^4 cells/vessel was listed first, and the experiment with the lowest response at the same seeding density was listed last. These analyses revealed that there was no consistent association of passage level of laboratory stock cultures with the level of the spontaneous transformation frequency among all ampules

of cells. Cell cultures derived from only 3 of 18 ampules (i.e., 1G, 1J, and 1N) exhibited a trend of increasing frequencies of spontaneous transformation with increasing passage level of laboratory stock cultures. In contrast, experiments using 15 of 18 ampules did not exhibit a clear trend of increasing frequencies with increasing passage level.

Culture Vessel Surface Area. The effect of culture vessel surface area on the magnitude of the frequency of spontaneous transformation was examined in three experiments (Figure 2). Data presented in Table 1 reveal that the level of the spontaneous transformation frequencies of cultures was essentially independent of the initial seeding density; however, all of these experiments used culture vessels with a surface area of about 27.8 cm^2. Thus, culture dishes were chosen for these experiments that had diameters of 35, 60, 100, and 150 mm that corresponded to total surface areas of 14.2, 27.8, 76.4, and 167.6 cm^2, respectively. The surface area for the contact-inhibited monolayer of cells included the culture dish bottom and 3.5 mm of the vertical edge. The volume of culture medium/vessel varied so that the height of the culture medium and cell growth was the same among all sets of dishes, and sets were seeded with 0.1, 0.32, 1.0, or 3.2×10^4 cells/vessel. These experimental conditions were selected because they resulted in large differences in the number of cell population doublings between small dishes seeded with high densities of cells and large dishes seeded with low densities of cells.

The spontaneous transformation frequencies of types I–II foci/vessel, as well as type III foci/vessel, are presented in Figure 2*A*,*B*, respectively. The largest culture dishes, with a diameter of 150 mm, had consistently high average transformation frequencies. For example, the four seeding densities of 3.2, 1.0, 0.32, and 0.10×10^4 cells/vessel in experiment 2 had 3.9, 2.5, 3.9, and 1.9 type III foci/vessel; in experiment 3 the same four seeding densities had 2.8, 2.7, 1.9, and 2.1 type III foci/vessel, respectively. In contrast, identical seeding densities in 35-mm culture dishes had much lower average transformation frequencies of 0.09, 0.23, and 0.40 type III foci/vessel. Furthermore, in these same experiments the intermediate size 60- and 100-mm dishes had intermediate frequencies. Thus, the magnitude of the type III focus spontaneous transformation frequencies was detected in proportion to the $\log_{10}$ of the culture vessel surface area. The magnitude of the type I–II focus spontaneous frequency was also detected in proportion to the $\log_{10}$ of the culture vessel surface area.

Effect of Seeding Density Crowding and Preexisting Transformants on Expression of Spontaneous Transformation

Analyses of the frequencies of spontaneous transformation described above revealed that the average transformation frequency of BALB/c-3T3 cells was relatively the same for cultures seeded with 0.32, 1.0, and 3.2×10^4 cells/vessel (Table 1). Nevertheless, further examination of these data revealed that the average frequency detected for cells seeded at 1×10^4 cells/vessel was frequently less than that of cultures seeded with a lower density 0.32×10^4 cells/vessel. Similarly, the average frequency detected for cells seeded at 3.2×10^4 cells/vessel was frequently higher than that of cells seeded at 1.0×10^4 cells vessels. These relatively minor variations in the level of the spontaneous transformation frequency with different seeding densities were attributed to either a seeding density crowding effect or to the presence of preexisting variants. Although the seeding density differences in the frequencies were usually not statistically significant, they were observed in most of the experiments (Table A1). Thus, these phenomena were investigated further in experiments using a larger range of seeding densities.

Seeding Density Crowding Effect. The expression of the spontaneous transformation of cultures seeded at 1.0×10^4 versus 0.32×10^4 cells/vessel was lower at the higher cell density (Table 2). The lower frequency of transformation at the higher seeding density was attributed to a crowding affect of the higher seeding density on the expression of the spontaneous transformed cell phenotype. A comparable trend in the transformation frequencies was observed in experiments 1–61 and 62–110; however, the crowding effects in both groups of experiments were not statistically significant. The crowding effect was investigated further in 13 experiments that used seeding densities of 0.1, 0.32, and 1.0×10^4 cells/vessel (Table 2), and experimental data from individual experiments are presented in Table A2. Data from these experiments showed that the average spontaneous transformation frequencies at the two lowest seeding densities were nearly identical. For example, the type I–III frequencies of vessels seeding with 0.1 and 0.32×10^4 cells were 1.55 and 1.54 foci/vessel. In contrast, the number of type I–III foci significantly declined to 0.64 type I–III foci/vessel at the highest seeding density of 1×10^4 cells/vessel.

Preexisting Spontaneous Transformants. The analyses of variability among the frequencies of spontaneous transformation described above also included an analysis of data for the presence of outlier observations (see Materials and Methods). These analyses revealed that the average frequency of type III or type I–III foci of cells from ampule 1N was a significant outlier ($p < 0.01$) from the remaining 17 ampules of cells (Table 1). In addition, the transformation frequencies in experiment 8 (ampule 1C), experiment 62 (ampule 1L), and experiment 77 (ampule 1N) were significant ($p < 0.01$) outliers from other experiments using the same ampule of cells (Table A1). For example, the frequency of type III foci in experiment 8 using cells from ampule 1C, for a seeding density of 3.2×10^4 cells/vessel, was 2.19 foci/vessel, and this frequency was significantly ($p < 0.001$) higher than the type III frequencies of experiments 9–11 of 0.05, 0.30, and 0.15 foci/vessel (refer to Table A1).

Experiments were performed to determine whether the elevated or outlier spontaneous transformation frequencies noted in individual experiments or in groups of experiments using a single ampule of cells were caused by the presence of preexisting transformants. In contrast to transformants that arose spontaneously during the course of the 4-week transformation experiment, preexisting

Table 2. Inhibition of detection of spontaneous transformation of BALB/c-3T3 cell by a seeding density crowding effect.[a]

Seeding density,[b] $\times 10^4$ cells/vessel	Frequency of spontaneous transformation,[c] foci/vessel, mean ± SE	
	Type I–III frequency	Type III frequency
Preliminary series of 13 experiments		
0.10	1.55 ± 0.20	0.43 ± 0.06
0.32	1.54 ± 0.19	0.45 ± 0.06
1.0	0.64 ± 0.18**	0.32 ± 0.08*
Extended series of experiments (1–61, FBS Lot A)		
0.32	1.61 ± 0.20	0.57 ± 0.06
1.0	1.20 ± 0.16 (NS)	0.45 ± 0.05 (NS)
Extended series of experiments (62–110, FBS Lot B)		
0.32	3.13 ± 0.64	1.34 ± 0.28
1.00	2.15 ± 0.51 (NS)	1.01 ± 0.24 (NS)

Abbreviations: FBS, fetal bovine serum; NS, not significant.

[a]A crowding effect on detection of spontaneous transformation occurred when WT cells seeded at a high and a low high seeding density had a lower spontaneous transformation frequency expressed at the higher seeding density. Presumably, the transformed cells had a more difficult time expressing the transformed phenotype at the higher seeding density.

[b]Laboratory cultures of different passage levels were trypsinized and cells were replated at seeding densities of 0.32, 1.0, and 3.2 $\times 10^4$ cells/60-mm dish (or 25-cm^2 flask).

[c]The number of spontaneous foci of different types of BALB/c-3T3 cells that were scored in the preliminary series of 13 experiments are provided in Table A2 and the extended series of 110 experiments in Table A1.

[d]Spontaneous transformation of BALB/c-3T3 cells resulted in a continuum of type I, II, and III foci of different sizes. The type I–III transformation frequency in this investigation included type I and II foci ≥ 1 mm in diameter and type III foci ≥ 2 mm diameter. The contribution of type I and II foci alone can be calculated by subtracting the frequency of type III foci from the frequency of type I–III foci.

*Significant crowding effect, $0.01 < p \leq 0.05$.

**Significant crowding effect, $p \leq 0.01$.

transformants arose spontaneously in the laboratory stocks of cells that supply cells for the transformation experiment. Since laboratory stocks of cells used to maintain the laboratory cultures and the transformation experiments were passaged separately, preexisting transformants could result in very high spontaneous transformation frequencies in either a single experiment, or in a group of experiments using a single ampule of cells.

The presence of preexisting transformed cells in laboratory stock cultures was investigated by measuring the frequency of spontaneous transformation in cultures seeded with a high range of seeding densities: 1.0, 3.2, and 10 $\times 10^4$ cells/vessel. The highest seeding density would presumably have the highest probability of detecting the preexisting transformants. In four experiments, the average spontaneous transformation frequencies of the 1, 3.2, and 10 $\times 10^4$ cell seeding densities were 0.59, 0.94, and 2.61 type III foci/culture vessel (Table 3). Thus, an increase of 3.2-fold in the seeding density from 3.2 to 10 $\times 10^4$ cells resulted in a 2.8-fold increase in the frequency of transformed foci. In contrast, a 3.2-fold increase in seeding density from 1.0 to 3.3 $\times 10^4$ cells/vessel resulted in only a 1.6-fold increase in the frequency of transformed foci. Therefore, these data suggest preexisting transformants of BALB/c-3T3 cells were a relatively small contaminant of most stock cultures of cells seeded at $\leq 3 \times 10^4$ cells/vessel.

Evidence for preexisting transformed cells was also investigated by retrospectively comparing the transformation frequencies of the highest seeding densities of 1.0 and 3.2 $\times 10^4$ cells/vessel in the 110 transformation experiments in this study. The data were averaged separately for the experiments that used the two different FBS lots. These data showed the same trend as observed in the four preliminary experiments, and the transformation frequencies at the 3.2 $\times 10^4$ seeding density were only 1.4- to 1.6-fold higher than the frequencies at the 1.0 $\times 10^4$ seeding density. Furthermore, the average frequency at the 3.2 $\times 10^4$ cells/vessel seeding density was not statistically

Table 3. Detection of preexisting spontaneous transformed cells[a] in cultures seeded at high cell densities.

Seeding density,[b] $\times 10^4$ cells/vessel	Frequency of spontaneous transformation,[c] foci/vessel, mean ± SE	
	Type I–III foci[d]	Type III foci
Preliminary series of four experiments		
10.0	ND	2.55 ± 0.14**
3.2	ND	1.10 ± 0.22 (NS)
1.0	ND	0.63 ± 0.14
Extended series of experiments (1–61, FBS lot A)		
3.2	1.94 ± 0.30*	0.66 ± 0.08*
1.0	1.20 ± 0.16	0.45 ± 0.05
Extended series of experiments (62–110, FBS lot B)		
3.2	2.91 ± 0.57 (NS)	1.37 ± 0.26 (NS)
1.0	2.15 ± 0.51	1.01 ± 0.24

Abbreviations: FBS, fetal bovine serum; ND, not determined; NS, not significant.

[a]There are two possible origins of spontaneous transformants of BALB/c-3T3 cells: preexisting transformants and transformants that occur spontaneously in the transformation experiment. Spontaneous transformants arising in the transformation experiment were detected at a low and relatively constant level in vessels seeded with 0.32–3.2 $\times 10^4$ cells (refer to Table 1). In contrast, preexisting transformants arose in laboratory cultures, and they are transferred with the WT cells to the transformation experiment. The preexisting transformant can be detected in culture vessels seeded with a relatively high cell seeding density of 10.0 $\times 10^4$ cells/vessel. Thus, elevated transformation frequencies detected at high seeding densities are indicative of spontaneous transformation frequencies containing preexisting, as well as spontaneously occurring transformants.

[b]Laboratory cultures of different passage levels were trypsinized, and cells were replated at seeding densities of 1.0, 3.2, and 10.0 $\times 10^4$ cells/60-mm dish (or 25-cm^2 flask).

[c]Due to the $\log_{10}$ distribution of the spontaneous transformed foci detected in these experiments (Fig. 1), the transforming activity of each culture vessel was first mathematically transformed to the $\log_{10}$ before the transformation frequency of individual seeding density sets of cultures was calculated. Thus, the mean number of transformed foci in this table is the anti-log of the $\log_{10}$ mean transformation frequency.

[d]Spontaneous transformation of BALB/c-3T3 cells resulted in a continuum of type I, II, and III foci of different sizes. The type I–III transformation frequency in this investigation included type I and II foci ≥ 1 mm in diameter and type III foci ≥ 2 mm diameter. The contribution of type I and II foci alone can be calculated by subtracting the frequency of type III foci from the frequency of type I–III foci.

*Significant increase in frequency of spontaneous transformation, $0.01 < p \leq 0.05$.

**Significant increase in frequency of spontaneous transformation, $p < 0.001$.

higher than the frequency at the 1.0×10^4 cells/vessel density (Table 3).

Average Frequency of Spontaneous Transformation of BALB/c-3T3 Cells

The data presented in Tables 1–3 and Tables A1 and A2 demonstrate that several different experimental parameters influence the magnitude of the frequency of spontaneous transformation detected for BALB/c-3T3 cells. The effects of these parameters on the response had to be considered in the process of estimating the average spontaneous transformation frequency in a standard transformation assay using 3.2×10^4 cells/vessel. Because the magnitude of the frequency was significantly affected by the ampule of cells used, and the number of experiments with different ampules of cells varied from 3 (ampule 1J) to 10 (ampule 10), the mean response of the 110 experiments was a biased estimate of the average spontaneous transformation frequency. Therefore, the median frequency detected in experiments using the 18 ampules was used to estimate the average frequency in a standard experiment. These data showed that the median frequency of BALB/c-3T3 cells was either 1.24 type I–III foci/vessel or 0.57 type III foci/vessel for cells seeded at 3.2×10^4 cells/vessel (Table 1).

Discussion

This investigation examined the affects of varying five different experimental parameters on the detection of spontaneous transformation of BALB/c-3T3 cells to determine the parameters that caused variable expression of spontaneous transformed cell phenotype. To accomplish this goal, all experiments were conducted with a single cryopreserved pool of p3 cells. Cells from each ampule were passaged biweekly, and a total of 110 experiments were performed over a 2-year period. Because other investigations have shown that frequencies of spontaneous transformation were depended on the lot of FBS (*32–34*), this investigation used only two large lots of FBS. The FBS lots were screened and selected because they supported similar levels of spontaneous transformation. Finally, all laboratory cell cultures in these experiments were kept in log-phase growth by biweekly passage of the cultures at low seeding densities. If laboratory cultures had been permitted to become confluent, the frequency of spontaneous transformation could have increased with the passage of cells due to the selective growth advantage of preexisting transformed cells in laboratory cultures (unpublished data). Nevertheless, the average type III spontaneous transformation frequencies among these experiments ranged from 0.06 foci/vessel in experiment 5 (ampule 1B) to 8.01 foci/vessel (≥ 2 mm in diameter) in experiment 82 (ampule 1N; Table A1).

The most important experimental parameter associated with variable expression of spontaneous transformation of BALB/c-3T3 cells was shown to be the use of different ampules of cryopreserved cells. The 18 different ampules had average type III focus transformation frequencies ranging from 0.17 foci/vessel (ampule 1C) through 4.63 foci/vessel (ampule 1N; Table 1). Ampules of cells that resulted in a low average transformation frequencies had consistently low frequencies detected among experiments using cells at different passage levels. Conversely, ampules of cells with high frequencies had consistently elevated frequencies in all experiments using these cells.

In contrast, several experimental parameters were shown to have either no effect or only a relatively small effect on the magnitude of the frequency of spontaneous transformation. The median spontaneous frequency detected in experiments using FBS lot B was about 2-fold higher than frequencies detected in experiments using FBS lot A, but there was no clear separation of FBS-dependent activities in all experiments (Table 1). Similarly, experiments from a few ampules of cells showed a trend toward increasing spontaneous transformation frequencies with increasing passage number; however, this trend was not observed for most ampules of cells. For example, cells from ampule 10 were used in experiments from p6 through p24, and the spontaneous frequencies remained constant with increasing passage (Table A1).

The magnitude of the frequency of spontaneous transformation was also shown to be relatively independent of the initial seeding density of the cell cultures. The average frequency for cell cultures seeded with 0.32 and 3.2 cells/vessel were nearly identical for different ampules of cells (Table 1) and in different experiments (Table A1). Although the spontaneous transformation frequency increased in cultures seeded with a high seeding density of 10×10^4 cells/vessel (Table 3), elevated frequencies were not consistently observed at the 3.2×10^4 cells/vessel seeding density, which is used in the standard transformation experiment (*11*). Thus, significant numbers of preexisting transformants were not observed in most experiments.

The expression of transformed BALB/c-3T3 cells can be suppressed by the presence of WT cells. Sivak (*35*) reported that WT BALB/c-3T3 cells suppressed the expression of SV-40 transformed BALB/c-3T3 cells. Furthermore, we have observed that the phenotype 3-methylcholanthrene (MCA)-transformed cells was suppressed by WT cells (unpublished observations). Likewise, Haber et al. (*36*) has demonstrated that WT C3H10T1/2 cells suppressed the expression of C3H10T1/2 cells transformed with MCA. Nevertheless, the expression of the transformed phenotype has been reported to be comparable for cultures seeded at different densities (*17,37–39*). Therefore, the restriction of the expression of the MCA-transformed cell phenotype was relatively independent of the initial seeding density of the cell cultures.

In this investigation, a small crowding effect was seen in cell cultures seeded at 1×10^4 cells/vessel (Table 2), versus cells seeded at lower density of 0.32 or 0.10×10^4 cells/vessel, but this effect was usually not significant, and it was not observed in all experiments (Table A1). Therefore, if the phenotype of spontaneous transformed cells was suppressed by the WT cells in these cell cultures, then the suppression of the phenotype must have relatively comparable for cultures seeded with $0.10–3.2 \times 10^4$ cells/vessel.

The abnormal distribution of types I–III foci/vessel of BALB/c-3T3 cells has been previously reported (*11,27,28*). In this investigation the frequency of spontaneous transformation data were converted to a normal distribution using a $\log_{10}$ mathematical transformation procedure (Fig. 1); thus, these results agreed with previous investigations from this laboratory (*11,27*). The biological cause of the abnormal distribution of spontaneous foci is unknown; however, it may have five or more possible explanations. First, spontaneous transformation may have been caused by activation of a latent transforming virus in BALB/c-3T3 cells (*17*). If this virus was activated in a few subconfluent cell cultures, it could have reinfected many growing cells in these cultures and thereby induced many transformed foci in a few culture vessels. Second, the early appearance of a spontaneous focus in a few cell cultures could have formed many sister colonies in the same culture vessel. The sister colony foci may have been formed when the parent focus was mechanically dislodged or broken during the maintenance feeding of the cultures. Third, the expression of the transformed phenotype in a culture vessel may depend on a critical size of the colony of spontaneous transformed cells (*17,36,40*), and the transformed phenotype of most foci might be suppressed by the WT cells. Thus, the kinetics of expression of the transformed phenotype, or conversely the suppression of the phenotype, could be abnormal due to a multistep process required for expression. Fourth, the expression of the transformed phenotypic might depend on the $\log_{10}$ concentration of a critical factor in FBS or a factor produced by the transformed cells. Finally, all four explanations for the $\log_{10}$ distribution of the transformed phenotype may be correct, and each mechanism could have resulted in culture vessels with large numbers of spontaneous transformed foci. Taken together, the cause of this phenomenon may be inexplicable in individual culture vessels, but the presence of these vessels does not prevent the focus data possessing a skewed distribution from being easily analyzed with parametric statistical procedures.

The mechanism(s) of spontaneous morphological transformation of the A-31-1-13 clone of BALB/c-3T3 cells is unknown and was not the objective of these investigations. Nevertheless, data from the literature, as well as data obtained in this investigation, are consistent with the hypothesis that a spontaneous transformed focus arose as a mutational event in a WT cell (*1,2,41,42*). According to this hypothesis, the phenotypic change from a WT to a spontaneous transformed cell was a relatively rare mutagenic change in the genotype of WT cells, which enabled the transformed cell to replicate within a contact-inhibited monolayer of WT cells. Several reports in the literature have documented that spontaneous transformation of BALB/c-3T3 cells represents a permanent phenotypic change in WT cells because isolated spontaneous transformed foci of BALB/c-3T3 cells have maintained the transformed phenotype through many passages (*12,21,26*). Nevertheless, the data in this study do not exclude the possibility that spontaneous transformation was caused by some other single step phenomena (*43*).

In this investigation the mutation hypothesis is supported by data that were obtained in experiments used to determine the appropriate method of calculating the frequency of spontaneous transformation of BALB/c-3T3 cells. To calculate this frequency, it had to be determined whether the frequency of spontaneous transformation was related to the cumulative number of cell mitoses in the cell cultures, the initial seeding density of cells, or the number of population doublings. This question was answered by performing an experiment in which culture vessels of different sizes were seeded with cells at different seeding densities. The data presented in Figure 2 clearly demonstrate that the frequency of spontaneous transformation of the cells increased in culture vessels in direct proportion to the $\log_{10}$ of the surface area of the vessel. In other words, the frequency of spontaneous foci was directly proportional to the number of cells that survived and proliferated to confluence. Because the number of cells per unit of surface area was identical in culture vessels of different sizes, the transformation frequency was directly proportional to the $\log_{10}$ of the culture vessel surface area in the confluent monolayer. Thus, the frequency of spontaneous transformation of BALB/c-3T3 was detected in proportion to the cumulative number of cell mitoses required to form the contact-inhibited monolayer. A culture vessel with a large surface area accumulated more cell mitoses to form a monolayer of cells than a culture vessel with a small surface area. Furthermore, the majority of cell mitoses in BALB/c-3T3 cultures occurred during the final cell divisions to form the monolayer. Therefore, cultures seeded with relatively low densities of $0.1–3.2 \times 10^4$ cells/60-mm dish would have had nearly identical cumulative mitoses to form a contact-inhibited culture and nearly identical chances to undergo a spontaneous mutation.

In contrast, in the same experiments using different size culture vessels seeded with different numbers of cells, the frequency of spontaneous transformation was not influenced by the initial seeding density of the cells. Likewise, the spontaneous transformation frequency was not related to the number of population doublings. Cells seeded at different densities required different numbers of population doublings to achieve confluency, yet they had equivalent frequencies of spontaneous foci in vessels with the same surface area. Comparable observations have been reported for the expression of MCA-transformed C3H10T1/2 cells (*17,37–39*).

Thus, the frequency of expression of spontaneous foci detected in the BALB/c-3T3 cells was shown to be comparable to that obtained for single gene mutation in mammalian cells (*41*). In this investigation the frequency for type III transformed foci that arose in cell cultures was estimated to be 0.71×10^{-6} for a contact-inhibited monolayer containing 8×10^5 cells/60-mm dish. This frequency was based on cultures seeded with 3.2×10^4 cells, in which a median frequency of 0.57 type III foci/vessel were observed. Similarly, the estimated frequency for the combined type I–III foci was 1.55×10^{-6}, which is based on the detection of a median frequency of 1.24 foci/60-mm dish that were seeded with 0.32×10^4 cells.

If one assumes that spontaneous transformation were a mutational event that occurred at a rate of 0.71×10^{-6} in these experiments, then one would predict that spontaneous mutations should occasionally have arisen in laboratory cultures of cells that were passaged to provide cells for transformation experiments. This prediction was apparently confirmed, because significant outlier frequencies of spontaneous transformation were detected in experiments 8 and 62. In these experiments, 2–5 laboratory cultures were passaged at $0.75\text{-}2 \times 10^5$ cells/100-mm dish resulting in 10–50 vessels with $1\text{–}6 \times 10^6$ cells/100-mm dish (i.e., total of $10\text{–}30 \times 10^6$ cells/experiment). Based on a type III focus forming frequency of 0.71×10^{-6}, a spontaneous transformant could have occurred in these cultures and could have increased in frequency due to their selective growth advantage over WT cells. Because these laboratory cultures were used in only one experiment, the preexisting transformants would have resulted in a high frequency in only one experiment using cells from the same ampule. Thus, experiment 8 was observed to have an outlier frequency compared to the frequencies of experiments 9–11 using cells from ampule 1C, and experiment 62 was an outlier compared to experiments 63–69 using cells from ampule 1L (refer to experiments C 8–11 and L 62–63; Table A1).

Because laboratory cultures were serially passaged to maintain a consistent source of stock cells for experiments, but these cultures were kept separate from the cultures used for the transformation experiments, one would also predict that a spontaneous transformation mutation occurring in an early passage laboratory cultures would result in an series of experiments with unusually high frequencies of spontaneous transformation. While laboratory culture stock cells were purposely passaged biweekly at a relatively low seeding density of 5×10^4 cells/100-mm dish, a rare mutation at a rate of 0.71×10^{-6} could have occurred nevertheless. Furthermore, if this mutation occurred, then the preexisting transformants could have progressively increased with serial passage of the cultures due to their selective growth advantage over WT cells. This type of preexisting transformant may have occurred in early passage cells derived from ampule 1N because the first experiment (no. 77) using low-passage laboratory culture cells had a frequency of 0.97 type III foci/vessel, and subsequent experiments 78–82 had frequencies of 3.28, 5.12, 3.02, 7.37, and 8.01 type III foci/vessel. As predicted, the frequencies of spontaneous transformation of experiments using cells from ampule 1N increased with passage of cells from ampule 1N. A similar phenomenon may also have occurred with experiments using cells from ampule 1J (Table A1). Taken together, the analysis of the data for outlier transformation responses demonstrated that unusually high frequencies of spontaneous transformation in a single experiment or in a series of experiments using related laboratory culture of cells were relatively rare and could be explained by a spontaneous mutation hypothesis. Likewise, these data also suggest that preexisting spontaneous transformants of WT cells in laboratory cultures were a relatively rare occurrence in this investigation.

In conclusion, the major finding of this study is that the magnitude of the spontaneous transformation of BALB/c-3T3 cells depends on the ampule of cryopreserved cells used in the experiments. Although the mechanism by which cells express different levels of spontaneous transformation is unknown, these data are consistent with a hypothesis that spontaneous morphological transformation of BALB/c-3T3 cells is a mutational event. Regardless of the mechanism by which this transformation occurs, the proportional effect of culture vessel surface area on the frequency of spontaneous transformation justifies the calculation frequencies of transformation as either mean foci/vessel or foci/cell in the confluent monolayer. In contrast, the frequency of transformation should not be expressed as the number of transformed foci per viable cell seeded. Thus, frequency of type III transformed foci in this study was estimated to be 0.71×10^{-6}. These observations are very important because the 110 experiments listed in this paper were also used to investigate the chemical-induced transformation responses of many different carcinogenic and noncarcinogenic chemicals [refer to part IV and V of this series (*25,44*)]. In addition, all of these experiments included a BaP positive control tested at two concentrations, and these data have been summarized and reported separately in part II of this series (*45*). Taken together, these experiments provide a large database to compare chemical-induced and spontaneous transformation responses of BALB/c-3T3 cells.

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Appendix A.

Table A1. Evaluation of frequencies of spontaneous transformation[a] of BALB/C-3T3 cells at three seeding densities in 110 experiments.

Experiment conditions[b]			Transforming activity versus seeding density[c] Seeding densities (x10⁴ cells/vessel)									Transformation frequency[d]					
			0.32			1.0			3.2								
			I&II	III	III	I&II	III	III	I&II	III	III	Type I-III foci			Type III foci		
Amp. no	Exp. no	Pass no.	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	0.32	1.0	3.2	0.32	1.0	3.2
1C	8[e]	4	41	16	15 (20)	51	19	17 (20)	136	48	62 (40)	3.11	3.52	4.94	1.26	1.39	2.19
	11	11	1	1	3 (20)	3	0	4 (20)	13	6	15 (40)	.15	.24	.38	.13	.15	.30
	9	6	3	0	1 (20)	1	4	4 (20)	7	4	4 (40)	.15	.27	.26	.04	.26	.15
	10	9	6	2	1 (20)	1	2	0 (20)	3	1	2 (40)	.35	.11	.10	.11	.07	.05
	AVG.											*.22**	*.21**	*.25**	*.09**	*.16**	*.17**
												.94	*1.04*	*1.24*	*.39*	*.47*	*.67*
1K	60	17	25	7	5 (20)	22	6	5 (20)	95	36	18 (40)	1.39	1.27	2.25	.39	.42	.77
	59	15	18	6	1 (19)	2	2	3 (20)	12	5	10 (40)	.84	.28	.53	.27	.19	.30
	57	11	6	7	3 (20)	8	2	1 (20)	28	7	8 (40)	.59	.44	.71	.39	.11	.28
	54	5	14	10	5 (19)	4	0	2 (19)	38	8	7 (40)	1.11	.23	.46	.62	.08	.27
	56	9	9	4	1 (20)	5	3	5 (19)	12	6	7 (39)	.53	.50	.51	.19	.32	.26
	58	13	20	6	3 (20)	18	4	3 (20)	11	4	6 (40)	1.15	.81	.39	.31	.28	.19
	55	7	0	3	1 (20)	2	1	0 (20)	5	5	2 (40)	.15	.11	.22	.15	.04	.13
	AVG.											*.78*	*.46*	*.44*	*.29*	*.18*	*.19*
1D	14	9	6	3	9 (20)	6	2	1 (20)	13	7	5 (40)	.70	.35	.37	.41	.11	.21
	13	7	14	4	1 (20)	22	5	2 (20)	7	5	6 (40)	.66	.42	.34	.19	.21	.20
	15	11	1	1	2 (20)	5	3	4 (20)	7	5	5 (39)	.15	.48	.31	.11	.25	.19
	12	5	35	4	5 (16)	12	1	3 (19)	10	4	4 (40)	1.60	.60	.31	.45	.14	.14
	AVG.											*.78*	*.46*	*.33*	*.29*	*.18*	*.19*
1L	62[e]	8	74	22	12 (20)	99	25	34 (20)	430	134	127 (40)	4.32	5.64	14.43	1.44	2.22	6.02
	63	8	12	7	2 (20)	14	3	8 (18)	91	36	48 (39)	.37	1.83	4.00	.70	.41	1.92
	69	16	7	4	5 (20)	2	2	3 (19)	17	3	12 (40)	.50	.27	.58	.26	.20	.39
	64	10	27	9	1 (20)	1	2	0 (20)	10	12	5 (40)	.77	.11	.48	.27	.07	.29
	65	12	12	6	3 (20)	8	6	4 (20)	14	10	4 (40)	.79	.63	.51	.28	.35	.24
	68	14	13	8	8 (18)	5	5	6 (18)	5	6	5 (36)	1.30	.70	.34	.74	.45	.23
	61	6	5	0	1 (20)	2	2	1 (20)	8	5	7 (40)	.20	.19	.37	.04	.11	.22
	67	14	2	1	5 (19)	2	2	1 (20)	6	3	2 (39)	.32	.17	.19	.32	.17	.09
	66	12	19	9	9 (20)	3	1	2 (20)	7	1	2 (38)	.82	.19	.19	.40	.11	.06
	AVG.											*.63**	*.51**	*.83**	*.38**	*.23**	*.43**
												1.04	*1.08*	*2.34*	*1.63*	*.45*	*1.05*
1B	6B	7	ND	8	10 (18)	ND	4	6 (36)	ND	11	6 (36)	ND	ND	ND	.53	.07	.51
	6A	7	ND	7	7 (20)	ND	2	0 (20)	ND	11	13 (38)	ND	ND	ND	.71	.45	.34
	7	9	2	1	1 (19)	2	3	7 (20)	6	6	1 (36)	.16	.44	.22	.08	.35	.14
	5	5	ND	1	1 (20)	ND	1	2 (20)	ND	2	0 (40)	ND	ND	ND	.07	.11	.03
	AVG.											*ND*	*ND*	*ND*	*.35*	*.25*	*.26*
1I	47	7	19	8	6 (20)	7	2	7 (20)	70	13	18 (39)	1.20	.61	1.19	.51	.33	.58
	48	9	36	7	15 (20)	5	2	1 (20)	34	11	18 (40)	1.60	.28	1.30	.64	.11	.54
	49	11	15	10	6 (20)	4	1	3 (19)	18	3	19 (40)	1.21	.32	.81	.53	.16	.43
	50	13	34	14	16 (19)	15	6	10 (20)	18	8	12 (39)	1.43	.85	.76	.84	.51	.38
	46	5	12	7	0 (20)	6	3	1 (20)	59	9	15 (40)	.73	.39	1.09	.27	.15	.38
	AVG.											*1.23*	*.49*	*1.03*	*.56*	*.25*	*.46*
1H	44	12	26	13	16 (20)	38	15	24 (20)	120	34	43 (40)	1.94	2.68	3.69	.99	1.55	1.52
	43	10	74	18	19 (19)	9	5	8 (19)	65	12	32 (35)	2.36	.82	1.81	1.13	.54	1.05
	42	10	15	3	11 (20)	6	3	4 (19)	27	17	35 (40)	1.13	.53	1.32	.51	.29	.86
	37	4	27	13	7 (20)	13	5	7 (20)	43	15	17 (39)	1.58	.77	1.51	.67	.45	.63
	40	8	5	5	9 (19)	19	6	3 (20)	17	11	17 (40)	.77	.61	.77	.62	.30	.53
	38	6	12	6	4 (20)	5	3	6 (20)	25	15	12 (40)	.78	.53	1.08	.35	.33	.50
	39	6	14	5	5 (20)	4	6	6 (20)	43	17	10 (40)	.88	.60	1.01	.37	.47	.43
	41	8	30	20	11 (18)	1	1	4 (18)	6	6	7 (36)	.83	.26	.41	.35	.21	.27
	45	14	5	3	2 (20)	5	3	1 (20)	18	1	6 (19)	.39	.37	1.09	.19	.15	.24
	AVG.											*1.18*	*.80*	*1.41*	*.58*	*.25*	*.46*

(Continued on next page)

Table A1. *Continued.*

Experiment conditions[b]			Transforming activity versus seeding density[c] Seeding densities (x10[4] cells/vessel)									Transformation frequency[d]					
			0.32			1.0			3.2								
			I&II	III	III	I&II	III	III	I&II	III	III	Type I-III foci			Type III foci		
Amp. no	Exp. no	Pass no.	>1	2-4	>4mm (*n*)	>1	2-4	>4mm (*n*)	>1	2-4	>4mm (*n*)	0.32	1.0	3.2	0.32	1.0	3.2
1E	22	13	26	6	7 (18)	22	29	34 (20)	66	18	27 (40)	1.67	2.21	2.35	.49	1.13	.87
	23	13	29	11	8 (18)	11	10	11 (18)	73	12	11 (27)	2.11	1.50	1.56	.83	.97	.66
	18	9	13	6	1 (20)	51	19	17 (20)	52	13	20 (40)	.74	.73	1.41	.26	.35	.59
	20	11	21	10	11 (20)	16	7	3 (20)	14	4	17 (40)	1.39	.94	.64	.77	.35	.37
	19	9	17	5	1 (20)	5	11	5 (19)	47	7	11 (38)	.84	.43	.70	.19	.39	.36
	17	7	14	9	4 (20)	16	12	7 (20)	46	8	10 (40)	1.12	1.15	.83	.47	.65	.35
	21	11	16	4	5 (19)	6	3	2 (19)	28	4	15 (40)	1.08	.45	.90	.33	.20	.34
	16	7	10	3	1 (18)	5	0	2 (18)	18	12	5 (36)	.51	.27	.73	.15	.08	.34
	AVG.											*1.18*	*.96*	*1.24*	*.44*	*.52*	*.51*
1F	26	9	99	11	11 (20)	73	4	9 (20)	332	31	10 (40)	5.69	3.89	8.90	.85	.50	.90
	28	11	18	4	3 (20)	20	4	3 (20)	83	23	10 (40)	1.01	.98	2.86	.23	.27	.82
	27	9	58	9	7 (18)	59	2	2 (18)	296	20	11 (36)	3.88	3.30	8.66	.74	.17	.66
	24	5	43	32	9 (20)	36	7	6 (20)	101	10	8 (40)	1.87	2.01	2.63	.56	.47	.31
	25	7	41	8	8 (20)	73	7	2 (40)	232	4	1 (40)	2.64	4.94	5.57	.74	.39	.10
	AVG.											*3.02*	*3.02*	*5.76*	*.62*	*.36*	*.56*
1O	90	20	52	25	41 (18)	24	15	33 (18)	178	110	109 (71)	3.77	2.59	2.93	2.53	1.92	1.95
	92	24	25	8	20 (18)	5	7	8 (18)	38	26	36 (71)	1.87	.81	.91	.96	.62	.60
	84	8	25	8	4 (18)	7	8	12 (18)	23	21	29 (72)	1.42	1.12	.72	.45	.81	.51
	89	18	14	6	5 (20)	3	3	7 (20)	33	21	36 (79)	.63	.46	.71	.32	.37	.49
	86	12	9	11	6 (18)	4	1	3 (18)	32	20	27 (72)	1.15	.31	.76	.75	.15	.46
	88	16	6	11	5 (18)	3	2	3 (18)	15	15	22 (67)	.96	.32	.58	.68	.19	.41
	87	14	17	8	4 (20)	3	1	5 (18)	29	24	19 (80)	1.19	.34	.60	.45	.24	.35
	83	6	9	5	1 (20)	18	4	2 (20)	30	24	24 (80)	.48	.78	.55	.23	.23	.35
	91	22	1	2	3 (20)	2	0	3 (20)	15	10	21 (75)	.21	.17	.46	.17	.11	.32
	85	10	10	8	7 (20)	7	1	2 (20)	32	23	15 (80)	.96	.50	.50	.59	.23	.31
	AVG.											*1.26*	*.74*	*.87*	*.71*	*.49*	*.57*
1Q	104	18	20	16	17 (18)	12	14	9 (18)	45	40	43 (71)	2.71	1.59	1.38	1.57	1.02	.88
	103	16	22	14	17 (20)	16	8	10 (20)	75	39	50 (79)	2.24	1.14	1.67	1.09	.58	.87
	102	14	10	8	9 (18)	5	5	9 (18)	41	39	25 (72)	1.23	.81	1.17	.71	.63	.70
	98	6	14	11	6 (18)	27	10	11 (18)	28	17	22 (47)	1.29	1.55	1.07	.71	.70	.62
	99	8	19	13	7 (20)	18	7	7 (20)	100	27	38 (80)	1.42	.89	1.04	.77	.53	.59
	105	20	31	14	4 (20)	13	3	5 (19)	50	30	28 (77)	.89	.78	.88	.36	.29	.58
	97	4	15	9	2 (20)	9	5	3 (20)	71	32	15 (80)	1.05	.67	1.07	.42	.32	.41
	100	10	13	8	8 (18)	2	5	4 (18)	16	7	21 (72)	1.29	.43	.42	.66	.35	.27
	101	12	10	5	5 (20)	7	0	2 (20)	28	12	15 (78)	.74	.37	.58	.35	.07	.26
	AVG.											*1.43*	*.91*	*1.03*	*.74*	*.50*	*.58*
1M	76	19	34	21	16 (18)	21	4	10 (18)	104	56	96 (71)	3.25	1.11	3.03	1.62	.53	1.79
	71	8	42	18	9 (20)	14	8	4 (20)	102	68	44 (75)	1.99	1.01	2.15	.93	.49	1.06
	75	16	52	17	8 (20)	10	7	2 (20)	136	49	40 (78)	3.33	.77	1.98	.89	.37	.88
	74	14	31	22	10 (18)	9	5	10 (18)	65	34	31 (71)	2.37	.92	1.17	1.00	.54	.66
	70	6	15	8	1 (6)	0	0	0 (3)	39	17	19 (54)	1.42	.00	1.07	.74	.00	.53
	72	10	24	10	11 (18)	12	4	3 (18)	29	10	19 (72)	2.15	.80	.62	.89	.29	.29
	73	12	22	5	3 (20)	37	11	7 (20)	54	25	20 (79)	1.30	2.54	.61	.27	.73	.27
	AVG.											*2.26*	*1.02*	*1.52*	*.91*	*.42*	*.78*
1A	4	9	ND	15	15 (20)	ND	7	15 (20)	ND	59	59 (40)	ND	ND	ND	.80	.70	1.51
	1	5	ND	10	12 (20)	ND	3	12 (20)	ND	30	43 (40)	ND	ND	ND	.73	.50	1.44
	2	5	ND	5	6 (20)	ND	5	6 (20)	ND	7	27 (40)	ND	ND	ND	.35	.42	.66
	3	7	ND	3	2 (20)	ND	0	2 (20)	ND	8	9 (40)	ND	ND	ND	.19	.07	.29
	AVG.											*ND*	*NA*	*NA*	*.52*	*.43*	*.98*
1P	95	9	109	32	15 (20)	49	17	13 (20)	389	141	122 (77)	5.96	3.36	6.83	1.79	1.27	2.84
	94	7	67	19	11 (18)	8	9	6 (18)	164	85	65 (71)	3.22	1.04	2.96	1.52	2.07	1.08
	96	11	55	6	4 (20)	35	2	1 (20)	97	32	30 (70)	2.86	1.10	1.74	.35	.11	.66
	93	5	33	22	4 (20)	16	12	16 (19)	27	18	25 (79)	1.65	1.91	.66	.84	1.19	.42
	AVG.											*3.42*	*1.85*	*3.05*	*1.13*	*1.16*	1.25

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Table A1. *Continued.*

Experiment conditions[b]			Transforming activity versus seeding density[c] Seeding densities (x10⁴ cells/vessel)									Transformation frequency[d]					
			0.32			1.0			3.2								
			I&II	III	III	I&II	III	III	I&II	III	III	Type I-III foci			Type III foci		
Amp. no	Exp. no	Pass no.	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	0.32	1.0	3.2	0.32	1.0	3.2
1G	34	12	65	27	19 (19)	39	21	14 (20)	148	56	62 (40)	5.38	3.34	5.76	2.15	1.34	2.51
	35	14	99	27	19 (20)	77	19	12 (20)	138	53	41 (40)	5.74	3.69	5.26	2.07	1.51	1.97
	32	10	76	20	12 (18)	101	22	11 (20)	128	36	55 (38)	4.37	3.14	4.54	1.29	1.09	1.97
	33	10	44	22	18 (20)	15	8	6 (19)	79	29	25 (37)	3.69	1.27	3.31	1.75	.62	1.04
	31	8	41	17	9 (18)	30	10	10 (18)	31	21	22 (36)	3.18	1.91	1.70	1.18	.91	.93
	30	6	12	9	1 (20)	21	9	6 (20)	45	19	21 (40)	.89	1.36	1.68	.37	.57	.68
	29	6	40	13	11 (20)	6	8	11 (20)	51	22	14 (40)	1.84	.96	1.02	.74	.70	.61
	36	14	9	3	2 (18)	5	3	2 (18)	21	10	10 (36)	.51	.45	.85	.19	.21	.42
	AVG.											*3.22*	*2.02*	*3.02*	*1.22*	*.87*	*1.27*
1J	53	9	161	24	24 (20)	104	13	11 (20)	464	79	49 (40)	5.33	3.76	9.73	1.34	1.00	2.76
	52	7	52	10	7 (20)	64	15	16 (20)	136	27	28 (38)	2.27	2.69	2.82	.69	1.19	1.09
	51	5	17	3	5 (19)	10	6	6 (19)	43	16	18 (40)	1.06	.86	1.64	.34	.45	.61
	AVG.											*2.89*	*2.44*	*4.73*	*.79*	*.88*	*1.49*
1R	107	9	90	19	12 (20)	43	10	18 (20)	655	180	94 (80)	5.08	3.16	9.56	1.19	1.07	2.95
	109	13	42	19	6 (20)	102	35	8 (20)	559	142	72 (80)	3.02	5.30	8.96	.89	1.11	2.55
	108	11	24	7	2 (18)	15	9	2 (18)	134	77	31 (70)	1.42	1.14	2.78	.34	.42	1.17
	106	7	9	3	2 (18)	9	8	3 (18)	76	48	26 (38)	.61	.51	2.73	.21	.29	1.30
	110	15	23	8	1 (18)	11	5	4 (18)	146	46	19 (75)	1.39	.78	2.29	.36	.35	.61
	AVG.											*2.30*	*2.18*	*5.26*	*.58*	*.65*	*1.72*
1N	81	17	265	108	51 (18)	150	79	89 (18)	704	276	307 (72)	22.4	17.3	15.3	8.27	9.05	7.37
	82	19	172	89	84 (18)	201	61	77 (18)	654	308	341 (72)	17.5	17.5	15.6	9.12	7.17	8.01
	79	12	142	70	71 (18)	75	43	45 (18)	350	211	219 (72)	15.1	8.02	9.40	7.53	4.32	5.12
	78	10	104	32	38 (18)	49	21	23 (18)	405	162	134 (72)	7.85	3.69	6.49	3.30	1.79	3.28
	80	14	153	62	37 (20)	72	28	19 (17)	255	138	179 (80)	12.0	5.61	5.83	4.60	2.20	3.02
	77[e]	8	45	17	7 (20)	53	11	8 (20)	152	58	36 (78)	2.89	2.51	2.32	.97	.78	.97
	AVG.											*13.0*	*9.11*	*9.16*	*5.63*	*4.22*	*4.63*

Abbreviations: Amp. no., ampule number; AVG., average; exp. no., experiment number; n = number of culture vessels; ND, not determined; pass. no., passage number of the cell cultures.

[a]Frequencies of spontaneous transformation: To facilitate comparisons of the effects of several different experiment parameters on detection of spontaneous transformation frequencies, the transformation assay data were rank-ordered. First, the average transformation frequency of type III foci of cells from the 18 different ampules were rank-ordered according to the frequency detected at the 3.2×10^4 cells/vessel seeding density. Thus, ampule 1C with the lowest average type III transformation frequency was presented first, and ampule 1N with the highest type III frequency was presented last. Second, individual experiments using cells derived from a single ampule were also rank ordered. The experiment with the highest total transformation frequency at the 3.2×10^4 cells/vessel seeding density was listed first, and the experiment with the lowest frequency was listed last.

[b]Experimental condition: Spontaneous transformants of BALB/c-3T3 cells were scored in 110 experiments numbered (Exp. no.) 1–110. All of the experiments used cells from one large pool of cryopreserved p3 cells. A total of 18 different ampules of cells labelled 1A through 1R were used to initiate laboratory cultures over a range of passages from p4 to p22.

[c]Transforming activity versus seeding density: Laboratory cultures of BALB/c-3T3 cells in log-phase growth were trypsinized and replated at 0.32, 1.0, and 3.2×10^4 cells/culture vessel. After a standard assay incubation period, culture vessels were fixed, stained, and evaluated for the presence of transforming activity (see Material and Methods). Large type III foci greater than 4 mm in diameter were distinguished from small type III foci that were 2–4 mm in diameter. The type I category of foci included types I and II foci ≥ 1 mm in diameter. The number of surviving culture vessels are shown within parentheses (n).

[d]Transformation frequency: Spontaneous transformation of BALB/c-3T3 cells resulted in a continuum of type I, II, and III foci of different sizes. The type I–III transformation frequency in this investigation included type I and II foci ≥ 1 mm in diameter and type III foci ≥ 2 mm diameter. The contribution of type I and II foci alone can be calculated by subtracting the frequency of type III foci from the frequency of type I–III foci.

[e]Outlier experiment: An outlier experiment in this investigation was defined as an experiment with a frequency of spontaneous transformation that was significantly different from other experiments that used cells from laboratory cultures from the same ampule (*27*). Three of the 110 experiments were determined to be outliers: experiment 8 using cells from ampule 1C, experiment 62 using cells from ampule 1L, and experiment 77 using cells from ampule 1N.

Table A2. Evaluation of frequencies of spontaneous transformation[a] of BALB/c-3T3 cells seeded at three different low-seeding densities in 13 consecutive experiments.

Experiment conditions[b]			Transforming activity versus seeding density[c] Seeding densities (x10⁴ cells/vessel)									Transformation frequency[d]					
			0.32			1.0			3.2								
			I&II	III	III	I&II	III	III	I&II	III	III	Type I-III foci			Type III foci		
Amp. no	Exp. no	Pass no.	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	>1	2–4	>4mm (*n*)	0.32	1.0	3.2	0.32	1.0	3.2
1I	P13	13	34	10	8 (20)	111	31	30 (60)	15	6	10 (10)	2.10	2.05	.85	.69	.65	.51
	P10	7	25	9	5 (20)	117	33	26 (60)	7	2	7 (20)	1.66	2.17	.61	.56	.60	.33
	P12	11	21	9	2 (20)	63	25	18 (60)	4	1	3 (19)	1.29	1.18	.32	.44	.48	.16
	P11	9	10	5	5 (20)	83	25	33 (60)	5	2	1 (20)	.83	2.00	.28	.39	.65	.11
	P 9	5	6	3	2 (20)	29	15	3 (60)	6	3	1 (20)	.42	.61	.39	.17	.23	.15
1J	P15	7	59	16	7 (20)	171	48	26 (59)	64	15	16 (20)	2.71	3.08	2.69	.76	.89	1.19
	P14	5	26	4	4 (20)	99	12	15 (59)	10	6	6 (19)	1.25	1.61	.86	.32	.30	.45
1K	P19	11	46	12	4 (20)	77	21	9 (60)	8	2	1 (20)	2.67	1.56	.44	.61	.59	.28
	P21	15	44	10	5 (20)	93	28	9 (59)	2	2	3 (20)	2.37	1.80	.28	.72	.46	.19
	P20	13	33	3	2 (19)	61	21	6 (60)	18	4	3 (20)	1.58	1.24	.81	.16	.34	.28
	P17	7	31	4	2 (20)	27	8	5 (60)	2	1	0 (20)	1.32	.49	.11	.19	.16	.04
	P18	9	21	4	3 (20)	55	13	6 (58)	5	5	3 (19)	1.14	1.07	.50	.28	.24	.32
	P16	5	13	5	2 (20)	70	30	19 (58)	4	0	2 (19)	.72	1.20	.23	.28	.50	.08

Abbreviations: Amp. no., ampule number; exp. no., experiment number; n = number of culture vessels; pass. no., passage number of the cell cultures.

[a]Frequencies of spontaneous transformation: To facilitate comparisons of the affects of several different experiment parameters on detection of spontaneous transformation frequencies, the transformation assay data were rank-ordered. First, the average transformation frequency of type III foci of cells from the 18 different ampules were rank-ordered according to the frequency detected at the 3.2×10^4 cells/vessel seeding density. Thus, ampule 1C with the lowest average type III transformation frequency was presented first, and ampule 1N with the highest type III frequency was presented last. Second, individual experiments using cells derived from a single ampule were also rank ordered. The experiment with the highest total transformation frequency at the 3.2×10^4 cells/vessel seeding density was listed first, and the experiment with the lowest frequency was listed last.

[b]Experimental condition: Spontaneous transformants of BALB/c-3T3 cells were scored in 110 experiments numbered (Exp. no.) 1–110. All of the experiments used cells from one large pool of cryopreserved p3 cells. A total of 18 different ampules of cells labelled 1A through 1R were used to initiate laboratory cultures over a range of passages from p4 to p22.

[c]Transforming activity versus seeding density: Laboratory cultures of BALB/c-3T3 cells in log-phase growth were trypsinized and replated at 0.32, 1.0, and 3.2×10^4 cells/culture vessel. After a standard assay incubation period, culture vessels were fixed, stained, and evaluated for the presence of transforming activity (see Material and Methods). Large type III foci greater than 4 mm in diameter were distinguished from small type III foci that were 2–4 mm in diameter. The type I category of foci included types I and II foci ≥ 1 mm in diameter. The number of surviving culture vessels are shown *within parentheses* (*n*).

[d]Transformation frequency: Spontaneous transformation of BALB/c-3T3 cells resulted in a continuum of type I, II, and III foci of different sizes. The type I–III transformation frequency in this investigation included type I and II foci ≥ 1 mm in diameter and type III foci ≥ 2 mm diameter. The contribution of type I and II foci alone can be calculated by subtracting the frequency of type III foci from the frequency of type I–III foci.